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Chapter 6

Neutron Reflectometry for Studying Proteins/Peptides in Biomimetic Membranes

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Additional information is available at the end of the chapter
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Abstract

The development of biomimetic surfaces for protein and peptide adsorptions is continuously expanding. Their biological functions can be influenced by the properties of the underlying artificial environment but the detailed mechanism is still not clear. In the past 30 years, neutron reflectometry has been widely applied to characterise the molecular structure of proteins or multi-protein complexes and their interactions with fluid artificial membrane that mimics the cellular environment. The specific interactions, bindings or structural changes between proteins and membranes play a crucial role in cellular responses and have promising potential in diagnostics and other biosensor applications. This chapter presents the progression of surface design for protein adsorption/interactions on membranes in detail, ranging from a simple phospholipid monolayer setup to more complicated artificial lipid bilayer systems. Furthermore, a new development of designed surfaces for studying the integral membrane protein system is also discussed in this chapter. A brief overview of various membrane mimetic surfaces is first outlined, followed by presenting specific examples of protein-membrane interactions studied by neutron reflectometry. The author demonstrates how to use neutron reflectometry as an advanced technique to provide step-by-step structural details for biomolecular applications in a well-controlled manner.

Keywords: Neutron reflectometry, biomimetic surfaces, artificial membrane, proteins, peptides

1. Introduction

Proteins are vital molecules that are responsible for many critical biological processes such as photosynthesis, respiration, cellular signal transduction, molecular transportation and enzymatic catalysis [1]. Most of these biochemical processes involve the interactions with proteins that are embedded in or associated with lipid molecules [2]. The protein-membrane
interactions are dynamic, complicated and mainly driven by hydrophobic and electrostatic interactions, but the detailed mechanism is still not clear [3]. Understanding and further modulating the interactions between proteins and membranes are essential for many biological and biotechnological applications. Many in vitro studies have been conducted to reveal and monitor the molecular interactions between proteins and lipids [3, 4]. The proteins are adsorbed or immobilised on a biomimetic surface that reconstitutes the physiological environment, i.e., the use of artificial membrane models to mimic the biological membrane [5].

Neutron reflectometry is a powerful and non-destructive technique that has been widely employed for studying biomolecular interactions. It has unique properties to provide quantitative structural and compositional details of the model interfaces without physical damage to the sample, which is impossible to achieve using other techniques [6, 7].

Neutron reflectometry is a powerful tool to probe the interfacial chemical structures at the microscopic level. The setup for neutron reflectometry measurements requires a parallel neutron beam that is incident onto the sample deposited onto an optically flat surface, where the neutron beams reflect and exit from the opposite end [6]. The neutron reflectivity, R, refers to the ratio of the incoming and exiting neutron beams and it is measured as a function of momentum transfer $Q$, which is defined as $Q = 4\pi \sin\theta / \lambda$, where $\theta$ is the angle of incident and $\lambda$ is the neutron wavelength [6, 8, 9]. $R(Q)$ is approximately given by $R(Q) = \frac{16\pi^2 Q^2}{Q^2 + 1} \rho(Q)^2$, where $R(Q)$ is the one-dimensional Fourier transform of $\rho(z)$, the scattering length density (SLD) distribution normal to the surface. SLD is the sum of the coherent neutron scattering lengths of all atoms in the sample layer divided by the volume of the layer. Therefore, the intensity of the reflected beams is highly dependent on the thickness, densities and interface roughness of the samples [4, 6]. If there are mixed layers containing two components, A and B and water (w), the scattering length density will be $\rho = \phi_A \rho_A + \phi_B \rho_B + \phi_w \rho_w$, where $\phi$ is the volume fraction of each layer and $\rho$ can be different contrasts from the same layer/substance, called contrast variations. The use of contrast variations in neutron reflectometry experiments allows resolving molecular species in response to stimuli; hence, it is possible to obtain detailed structure information with very high resolution [9]. There are two different approaches for contrast variations: magnetic contrast and isotopic contrast. Polarised neutron can generate a magnetic contrast that is able to produce simultaneously two datasets from exactly the same layer without changing the chemical environment [10, 11]. On the other hand, the isotopic approach is often used in the characterisation of multilayer, biological films [7]. By changing different buffer compositions ($D_2O$, $H_2O$ and $D_2O/H_2O$ mixtures) or using selective deuteration to replace hydrogen in desire locations, the contrast of scattering density between the hydrogen and deuterium reveals the relative locations in multi-component systems [12, 13]. By monitoring the isotopic labels, the distributions of different components in an in-situ environment can be identified; hence the accurate compositional depth profiles and thickness of the biological film can be determined [4, 14]. Typically, this method is applied to investigate the structural orientation and conformation changes of proteins adsorbed on membranes in a non-crystalline aqueous environment [12, 13]. The structural information of proteins/or peptides and membranes can be determined at Angstrom nanometre scale.

Researchers have developed different artificial membrane models to reconstitute the essential features of the biological membranes, providing an appropriate system to investigate the
structure-function relationships of proteins in membrane-mediated processes such as protein- or peptide-membrane interactions [15, 16]. This review discusses and examines several protein adsorption studies on different biomimetic membrane models used in neutron reflectometry. The biomimetic membrane models used are phospholipid monolayer at air/water interface (Section 2), phospholipid monolayer on solid supports (Section 3) and fluid lipid bilayers on a solid support (Section 4). The preparation of each membrane model is briefly described and characterised by neutron reflectometry, with particular attention on their biological applications in protein/or peptide adsorption. Furthermore, there are reviews that describe a direct immobilisation of membrane proteins on gold-modified surface (Section 5), as an alternative method for protein adsorption studies. This method is suitable for the use of various immobilising surfaces and addresses the problem of maintaining the proper orientation of the immobilised proteins, hence should have a wide range of \textit{in vitro} applications. The summary of the key and significant findings is stated.

2. Biomolecules interact with phospholipid monolayer at air/water interface

Phospholipid monolayer at the air/water interface has been used as the simplest biomimetic membrane model [17]. A lipid monolayer can be formed by depositing the lipid solution on the surface of water within a Langmuir-Blodgett trough. The amphiphilic properties of the lipid molecules allow the hydrophilic headgroups to interact with the water and leaving the hydrophobic alkyl chains exposed to the air, forming a planar, two-dimensional lipid phase. The resulting homogeneous lipid monolayer is called a Langmuir monolayer with high reproducibility [17, 18] (Figure 1). The lipid packing density of the phospholipid monolayer formed using the Langmuir trough can be precisely monitored by controlling the applied surface pressure ($\pi$) as a function of the molecular area ($A$) at a constant temperature. At a low surface pressure, the disordered phospholipid molecules exist in the gas phase. When a lateral surface force is applied, the phospholipid molecules are compressed into the liquid-expanded phase, followed by the coexistence of the liquid-expanded and the liquid-condensed phases. Further increase in the surface pressure allows the phospholipid molecules to orient in an orderly manner and presents as the solid state. Once the applied pressure reaches a certain limit, the phospholipid monolayer collapses [17, 19]. The organisation and dynamics of the phospholipid monolayer at the air/water interface have received immense interests in their biological applications. This model setup allows us to tailor the lipid packing density, which has a direct influence on the protein or peptide interactions with membranes [18]. The ability to monitor the lipid packing density throughout the experiment is beneficial and unique about this biomimetic membrane, which is not easy to control in many artificial membrane models [18].

The physical properties of phospholipid monolayer at the air/water interface influenced protein adsorption, as demonstrated in Maierhofer and co-workers’ neutron reflectometry study [20]. The zwitterionic phospholipid monolayer used in the study consisted of 1,2-dipalmitoyl-sn-glycerol-3-phosphocholine (DPPC) mixed with various molar ratios of 1,2-dimyristoyl-sn-glycerol-3-phospho(1’-rac-glycerol) (DMPG), to evaluate the surface charges...
of phospholipid monolayer in affecting cytochrome c adsorption. Higher protein adsorption was observed on the DPPC monolayer mixed with a 30 molar percent of DMPG, for which the DPPC/DMPG monolayer, existed in liquid-condensed phase. However, similar amount of cytochrome c was adsorbed on the equal molar mixture of DPPC and DMPG monolayer, known as an ideal lipid mixing [20]. The result strongly suggested that the electrostatic interactions between cytochrome c and the DPPC/DMPG monolayers were driven by the domain formation during phase transition of the non-ideal lipid mixing of the phospholipid monolayer that is predominantly at the liquid-condensed phase rather than the liquid-expanded phase [20].

Miano et al. [21] observed a constant thickness of 16 Å when DPPC monolayer was deposited on the air/water interface by applying various surface pressure using Langmuir-Blodgett trough. The molecular density (area per molecule) varied from 40 to 70 Å² accompanying in the change of surface pressure [21]. The neutron reflectometry study from Miano et al. also showed that the amount of protein associated with membranes decreased when the lipid monolayer surface pressure increased. Another study investigated the interactions of lactoferrin, a typical tear protein, on the DPPC monolayer deposited at the air/water interface, as a model of precocular tear film outer interface. The amount of lactoferrin coadsorbed onto the DPPC monolayer decreased as the surface pressure on the DPPC monolayer increased [21]. The coadsorbed lactoferrin penetrated into the phospholipid monolayer and characterised into two layers: 20% of the protein phase was located at a top dense sublayer (15 Å) and 10% of the protein phase was adsorbed to the bottom sublayer (60 Å) [21]. The structural distribution of the coadsorbed lactoferrin layers provided a mechanistic detail of how the tear proteins interact with the lipid film, as an in vitro precocular model for evaporative tear loss [21].

Recently, the use of Langmuir trough in combination with neutron reflectometry also illustrated how packing density could be altered and resulted in different conformations of the proteins [18, 22]. Pirrone et al. [18] monitored the membrane association process of human immunodeficiency virus-1 Negative Regulatory Factor (HIV-1 Nef) protein on a phospholipid monolayer formed on air/water interface [18]. The conformation of HIV-1 Nef proteins was found to be sensitive and dependent on lipid packing density. At a high lipid packing density (35 mN/m), the HIV-1 Nef protein maintained in a compact conformation and did not insert into the phospholipid monolayer. In a low lipid packing density (20 mM/m), both n-terminal region and c-terminal unstructured loop of the HIV-1 Nef proteins underwent conformation changes [18, 22]. The repositioning of HIV-1 Nef proteins caused the hydrophobic residues on the phospholipid hydrophobic head group interacts with the aqueous solution, leaving the phospholipid hydrophobic tails exposed to the air. Protein adsorption occurs from the aqueous phase to the phospholipid monolayer.

Figure 1. Diagram of phospholipid monolayer deposited on air/water interface prepared in a Langmuir trough. The phospholipid hydrophilic head group interacts with the aqueous solution, leaving the phospholipid hydrophobic tails exposed to the air. Protein adsorption occurs from the aqueous phase to the phospholipid monolayer.
the N-terminal inserted into the membrane and the proteins were oriented away from the phospholipid monolayer to a position 70 Å away [18, 22].

3. Biomolecules interact with phospholipid monolayer on solid supports

The amphiphilic nature of lipid molecules allows a spontaneous formation of Langmuir-Blodgett lipid film on a solid support, including glass, silica, micas and metal substrates [17]. However, the hydrophobic tail of lipid molecules does not encourage the formation of lipid monolayer on hydrophilic substrates [23, 24]. The technical challenge is overcome by chemically grafting the solid support with a polymer or surfactant, for example, octadecyltrichlorosilane. The surface hydroxyl groups of octadecyltrichlorosilane form covalent siloxy bonds with the substrate, resulting in a self-assembled layer and leaving the hydrophobic layer for subsequent phospholipid adsorption. The strong hydrophobic interactions between the lipid molecules and the robust hydrophobic part of surfactant allow for the phospholipids to be deposited using a Langmuir-Blodgett technique and hence promotes the formation of homogeneous monolayer (Figure 2) [23, 24].

![Figure 2](http://dx.doi.org/10.5772/62781)

Figure 2. Phospholipid monolayer deposited on chemically grafted solid support, the hydrophobic interactions forms the phospholipid monolayer onto the self-assembled hydrophobic octadecyltrichlorosilane-modified surface.

The surface coverage of octadecyltrichlorosilane formed on the solid support was found to be 68%, with the thickness of 24.0 Å to 33.5 Å [24, 25]. Subsequently, phospholipid monolayers were deposited on the octadecyltrichlorosilane-modified solid support and their structural information was extracted from neutron reflectometry (Figure 2). Kuhl et al. [23] claimed that the structure of phospholipid monolayers was not affected by the quality of octadecyltrichlorosilane deposited underneath. The octadecyltrichlorosilane-lipid layer was further studied in detail by Hollinshead et al. [24]. They observed that the phospholipid molecules penetrated into the octadecyltrichlorosilane layer and reported as a four-layer model. The first layer was the solid support (22.6 Å); the second layer (17.9 Å) and the third layer (12.6 Å) consisted of a combination of octadecyltrichlorosilane and phospholipid hydrocarbon chains and the top layer (9.4 Å) contained the phospholipid headgroup molecules only [24]. On the other hand, Kuhl et al. also demonstrated that the phospholipid monolayer, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE), could be functionalised with polymer such as polyethylene glycol (PEG). The DSPE-PEG monolayer gave the thickness of 52 Å on quartz surface and 48
Å on silicon surface. Thickness fluctuations of the PEG layer were adjusted by varying the concentration of polyethylene glycol in the phospholipid monolayer [23].

Lu et al. [26] reported the formation of phospholipid monolayer, phosphorylcholine (PC), on octadecyltrichlorosilane had a thickness of 18 Å [26]. The use of phospholipid monolayer mimics one leaflet of a biological membrane, and therefore it is ideal to investigate the interactions of peripheral membrane proteins [18]. The membrane interactions between lysozyme and albumin have been extensively studied and well-characterised [26, 27]. These proteins were selected as the model proteins for undertaking the neutron reflectometry experiments to reveal their interfacial structural conformations when adsorbed onto the phospholipid monolayer. The adsorption of lysozyme and albumins on the phosphorylcholine monolayer formed a thick and diffuse layer with the thickness of 60 Å and 80 Å, suggesting a loose protein layer deposited on phosphorylcholine monolayer [26, 27].

The other application of phospholipid monolayer on a solid support is to investigate the sensitivity of biodiagnostic assays [26] showing non-specific interaction of protein molecules. To retain the protein bioactivity, the phospholipid monolayer can be functionalised with a ligand such as biotin that is widely used to control protein orientation during adsorption [28]. Biotin bound to the headgroup of the phospholipid molecules and formed a biotin-lipid layer, followed by the binding of tetrameric protein, streptavidin, on the biotin-lipid layer. Neutron reflectometry results from Schmidt et al. [28] showed that only 5% of the DPPC molecules were carrying a biotin moiety. The layer thickness of DPPC monolayer, biotin moiety and the streptavidin proteins were 24 Å, 10 Å and 41 Å, respectively [28], that can be well defined by neutron reflectometry.

4. Biomolecules associated with fluid lipid bilayers on a solid support

The use of fluid lipid bilayer membrane as a model platform to study the influence of the bilayer microenvironment in protein-membrane interactions has been rapidly developed [15, 16]. Various lipid bilayer models exist, including (a) single supported lipid bilayers, (b) tethered lipid bilayer membranes, (c) polymer-cushioned bilayer membranes and (d) floating supported bilayers as described in Figure 3 [29].

![Figure 3](image_url)

**Figure 3.** Different types of supported lipid bilayers on a solid support: (a) single supported lipid bilayers, (b) tethered lipid bilayer membranes, (c) polymer-cushioned bilayer membranes and (d) floating supported bilayers.
4.1. Single supported lipid bilayers

Single supported lipid bilayers (SLBs) are commonly employed as the simplest lipid bilayer model in which the lipid bilayers are directly deposited on a hydrophilic surface (Figure 3a) [29]. SLBs are the most popular artificial membrane model because of their long-term stability, ease of formation and manipulation and wide applicability in studying membrane-related features and applications [29]. The following three methods are often used individually or in combination to the formation of SLBs: lipid vesicle fusion, lipid-detergent method and Langmuir-Blodgett and Langmuir-Schaefer deposition [29].

4.1.1. Lipid vesicle fusion

Lipid vesicle fusion is a spontaneous reaction, where the lipid vesicles adsorb, rupture and form a lipid bilayer which covers greater than 80% of a solid hydrophilic support [30]. This process is dependent on the surface properties (charge, structure and roughness), lipid nature (composition, charge, size and physical state) as well as the nature of the solvent (pH and ionic strength) [29, 31, 32]. The lipid vesicle fusion technique was used for studying the formation of SLBs made of different types of unsaturated phospholipids, including 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (POPG), 1-palmitoyl-2-oleoyl-sn-glycero-3-[phospho-l-serine] (POPS), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-sn-glycero-3-phospho-l-serine (DOPS) [33–35]. The experiments were set above the phospholipid phase transition temperature, thus the lipid bilayers existed in the fluid phase. The formation of SLBs is symmetric between the two lipid layers, and the membrane structure can be modelled from the neutron reflectometry data [33–35]. The thickness of each layer varied depending on the types of phospholipids used to form SLBs. The lipid bilayer had an overall thickness ranging between 38 ± 3Å and 56 ± 3Å, which were separated into three discrete layers: head group one (in contact with bulk solvent), acyl tails and head group two (in contact with the solid support). The modelling data also yielded information on other structural parameters including area per lipid molecule, volume fraction and surface excess of the three discrete layers (head-tail-head) [33–35].

The integrity of SLBs formed by lipid vesicle fusion technique has been fully characterised using neutron reflectometry, and it has been shown as a promising application to study membrane-mediated processes. Fernandez et al. [36] studied the interactions of antimicrobial peptide, maculatin 1.1, on both eukaryotic- and prokaryotic-mimic membranes using neutron reflectometry. The cationic maculatin 1.1 showed a minimal effect to the eukaryotic-mimic membrane; whereas, a profound effect was found when interacting with the anionic head group on the prokaryotic-mimic membrane. A decrease in anionic head group thickness was observed, but the thickness of acyl tails region remained the same, suggesting a reordering of bilayer occurred to associate with transmembrane pore formation [36]. Hellstrand et al. [34] also used neutron reflectometry to determine the position of α-synuclein, an amyloid protein associated with Parkinson’s disease, within and outside of the membrane bilayer. The α-synuclein was found to embed in the bilayer outer head group via electrostatic interactions but did not penetrate into the hydrophobic acyl chain region. The study then compared the adsorption of α-synuclein on different biological relevant membranes which were composed
of pure POPC, the mixtures of POPC/POPS or cardiolipin. The effect of membrane fluidity (acyl chain saturation), head group separation and the electrostatic shielding of the phospholipids strongly influenced the association of α-synuclein onto the membranes [34]. An analogue study was carried out on prion protein, which is widely recognised as the causative agent for Parkinson’s disease when the protein is misfolded [33]. The interactions of N-terminal cleavage fragments (N1 and N2) of prion protein on negatively charged POPG-contained SLBs were probed using neutron reflectometry. The results implied that both N1 and N2 were inserted into phospholipid head groups; however, the interactions were stronger for N1 fragment because of the additional metal ion binding site located at a polybasic region. The insertion of N1 fragment induced an increase in lipid order in the absence of phase transition, as evidenced by lengthening of lipid acyl tails and decreasing in lipid area. It is possible that prion protein N-terminal fragment plays a functional role on membrane interactions [33]. Recently, Lu et al. [35] employed neutron reflectometry to define the molecular determinants of cellular protein layers adsorbed on mimetic apoptotic membranes. The interactions between annexin V and membranes contained different amount of phosphatidylserine (PS) phospholipids were examined, which is a crucial process to detect early/mid-stage cell membrane apoptosis in vivo and in vitro. The membrane properties that were affected by PS contents strongly affected annexin V binding, showing an increase in annexin V binding with an increase in PS content in the membrane and that there was a maximum binding at 20% of PS in the membrane. A decrease in the thickness of the adsorbed annexin V layer was observed with a 33% of PS content in membrane, suggesting the annexin V might alter its conformational change at high PS content [35].

4.1.2. Lipid-detergent method

Lipid-detergent method is an alternative method for depositing SLBs on solid support. Tiberg et al. [37, 38] have successfully demonstrated the formation of SLB with the aid of detergent incorporated into the lipid forming micelles. The presence of detergents allows saturated fatty acids such as DPPC that has high hydrophobicity, to form stable micelles in aqueous solution [39]. In this method, the detergent is first incorporated into the lipid solution in excess amount, this helps to emulsify the lipid molecules and raise to the critical micelle concentration. Once lipid molecules concentration reached the critical micelle concentration, they aggregated into stable lipid micelles, which were introduced and adsorbed onto a hydrophilic silica surface [37–40]. The resulted SLB was identical to those formed using the vesicle fusion method once the detergent was completely removed [29, 38, 40, 41]. The cholesterol incorporated DPPC membranes were also used to study the effect of added components on the resulting SLBs. The neutron reflectometry results showed that the cholesterol was located below the lipid head group region and subsequently led to an increase in membrane thickness [38].

In addition to the membrane structural information, neutron reflectometry was also used to study protein interactions with membranes [29, 42]. Vacklin et al. [42] investigated the physical interactions between phospholipase A₂ (PLA₂) upon regulating its enzymatic hydrolysis on supported lipid bilayer. They were able to monitor the membrane bilayer changing its morphology when exposed to PLA₂ and the reaction was irreversible. By using phospholipids
with a different degree of saturation, the rate of PLA₂ hydrolysis decreased as lipid saturation increased. This gave the smallest penetration depth on the unsaturated phospholipid, 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC); and the greatest penetration depth on the saturated phospholipid, DPPC [42]. Shen et al. [43] studied the interactions of lipopeptide surfactin on supported DPPC bilayers, observing that the membrane stability was highly dependent on the critical micelle concentration of surfactin. The membrane was stable at a low surfactin concentration (below the critical micelle concentration). Neutron reflectometry can further identify the accumulation of surfactin on membranes showing that the surfactin penetrated into the outer leaflet of the membrane bilayer with pore formations on the membrane. When the surfactin concentration reached or exceeded the critical micelle concentration, the DPPC SLB was solubilised and progressively removed from the surface [43].

4.1.3. Langmuir-Blodgett and Langmuir-Schaefer deposition techniques

Langmuir-Blodgett and Langmuir-Schaefer deposition techniques are the most versatile methods for the preparation of thin and highly organised molecular films [44]. The amphiphilic lipid molecules have the self-assembled properties, which allow the lipid molecules to arrange in a monolayer film at an air/liquid interface on a solid support. The vertical lipid deposition usually forms a homogeneous layer with a controllable thickness of the deposited film, also called Langmuir-Blodgett film [45]. The solid support is placed vertically into the lipid solution. The resulted monolayer (first layer) has the lipid head groups interacting with the solid support and the acyl tail regions pointing towards the gas phase on a hydrophilic solid support, whereas on a hydrophobic solid support, the acyl tail regions interact with the solid surface, leaving the lipid head groups point outwards. Another similar method, Langmuir-Schaefer deposition in which the solid support is oriented horizontally is commonly used for lipid deposition [45]. Combining both vertical and horizontal lipid deposition techniques, the formation of SLB can be easily achieved using the Langmuir-Blodgett method for depositing the first lipid layer, and the Langmuir-Schaefer method for the deposition of the second lipid layer [45].

Recently, Clifton et al. [46] demonstrated how the Langmuir-Blodgett and Langmuir-Schaefer methods can be used to create a asymmetric SLB as a mimic of the complex Gram-negative bacterial outer membrane. The inner membrane leaflet of the outer membrane is mainly composed of phosphatidylycholine, which was deposited on hydrophilic surface using the Langmuir-Blodgett method. The outer membrane leaflet of outer membrane was deposited via Langmuir-Schaefer method, which contained either Lipid A or Escherichia coli rough lipopolysaccharides [46]. In the combination of both Langmuir-Blodgett and Langmuir-Schaefer techniques, the asymmetric SLBs were detected with greater than 90% surface coverage on solid support. The asymmetric SLBs also maintained high stability over time; this enabled us to clearly resolve the structure of the asymmetrical bilayer leaflets [46]. The development of functionalised biomimetic membrane using Langmuir-Blodgett and Langmuir-Schaefer methods is of great interest in nanobiotechnology for years [45]. However, the applications of neutron reflectometry to study biological phenomenon on Langmuir-Blodgett and Langmuir-Schaefer method-formed SLBs are not available.
4.2. Tethered lipid bilayer membrane

Tethered lipid bilayer membrane (t-BLM) is the lipid bilayer deposited on molecular anchors that act as spacers between the membrane and the solid support [47]. The use of self-assembled molecules as a surface coating technique has been developed since the early 1990s [47]. Molecules or ligands that form spontaneously via chemisorption from a solution or gas onto a solid support can result in a self-assembled monolayer [47]. Short oligomers or alkanethiols are often used as the applications of self-assembled monolayer that forms covalent bonds to the solid support such as gold and mercury, and the terminal functional groups create new surface properties on the solid support [15, 47, 48]. The deposition of lipid vesicles onto self-assembled monolayer allows the formation of tethered lipid bilayer membrane in which the lower bilayer leaflet interacts with the terminal functional group on self-assembled monolayer and the upper leaflet of phospholipids forms a functional lipid layer (Figure 3b) [15]. Tailoring the surface properties of the solid support improves the long-term stability for t-BLM, and hence widening the potential applications.

A neutron reflectometry study from Junghans et al. [49] used the selection of spacer group in affecting the formation of tethered lipid bilayer membrane. The types and the length of the spacers influenced the molecular geometry of the lipid molecules and hence affecting the structural and electrical properties on the membranes [49]. The use of long alkyl chain spacers increased water incorporation within the spacer units, which gave rise to a less dense packing order, an increase in both bilayer defect rate and roughness of the bilayer interfaces [49]. Alternatively, shorter spacer units had the highest surface coverage and also little water incorporation, resulting in an increase in membrane resistances [49]. With the controlled length of spacer, an optimum membrane architecture can be created, which allows for creating the physiological environment in which the protein-membrane interactions can be studied and further leading to possible biological applications.

Recently, the applications of tethered lipid bilayer membrane and neutron reflectometry were employed to investigate the orientations and conformations of membrane-bound protein, glucocerebrosidase (GCase), in an aqueous environment [50]. GCase was partially inserted into the tethered lipid bilayer membranes with its active site exposed to the membrane-water interface [50]. The use of selective deuteration techniques and the reflection of neutrons simultaneously extended the applications for protein complex characterisation. The results obtained from neutron reflectometry showed a large conformational change in GCase when interacted with α-synuclein (α-syn) [50]. Previous research has suggested that the formation of α-syn/GCase complex inhibited the enzymatic reaction of GCase, which might lead to the development of Parkinson disease and related disorders [51].

4.3. Polymer cushioned membranes

Polymer cushioned membranes are lipid bilayers deposited on a soft polymeric layer that coated on a solid support (Figure 3c) [16]. The use of polymeric layer mimics the cytoskeleton or extracellular matrix in plasma membrane, which is important in reconstituting the morphology of membrane domains as well as maintaining the nature and mobility of the transmembrane proteins [16, 29]. The polymeric layer must have the following properties: the
hydrophilic polymer is able to form a thin, uniform layer for the deposition of lipid bilayer. And secondly, the polymeric layer has a well-defined elastic modulus that is highly reproducible and chemically inert to the membranes or to the solid support [16]. The thickness, density and water content of the polymer cushioned layer as well as the membrane bilayer can be determined by neutron reflectometry. The deformability of polymer cushioned membranes provides a more natural cell-like environment for membrane-associated process [52]. The experimental setup of neutron reflectometry allows the lipid bilayer to maintain at a physiological condition where the membrane exists in the fluid state. This provides a great potential to study and the membrane-mediated interactions and monitor the experimental conditions in situ [53].

The use of beta-amyloid peptides as the model for Alzheimer’s disease has been widely studied and recognised [54]. Dante et al. [53] used neutron reflectometry to study the role of beta-amyloid peptides on polymer cushioned membranes. The data from neutron reflectometry evidenced a change in the lateral diffusion of the lipid bilayer when exposed to beta-amyloid peptides. It, therefore, suggested that the beta-amyloid peptides penetrated into the lipid bilayer and softened the lipid membrane bilayers [53]. These results strongly indicated that the change in lipid membranes’ structural and mechanical properties could influence and alter membrane-based signal transduction. Monitoring the interactions of beta-amyloid peptides on membrane with neutron reflectometry provided insights into the onset of Alzheimer’s disease [53].

4.4. Floating supported bilayers

Floating supported bilayers were first described by Fragneto et al. [55] as a new artificial membrane system formed on a solid support. In this SLB model, the lipid bilayer was formed in two steps: a Langmuir-Blodgett technique and a Langmuir-Schaefer technique. Firstly, a Langmuir-Blodgett technique was used to deposit a monolayer of lipid onto the solid support that was placed vertically (lipid monolayer with dark blue headgroup on Figure 3d). A lipid bilayer (lipids with light blue headgroup) was then horizontally introduced onto the lipid monolayer using a Langmuir-Schaefer technique (i.e., horizontal deposition). Therefore, the second bilayer interacted with the lipid monolayer, hence named floating lipid bilayer (lipid bilayer with light blue headgroup on Figure 3d) [55].

Talbot and co-workers [41] used neutron reflectivity technique to compare the formation of SLBs using various methods as described previously, this was further extended to investigate the interactions between a cationic gene delivery vectors (lipoplexes) with different SLB model membranes such as direct membrane deposition and polymer cushioned membranes. The data showed that the cationic lipoplexes could easily destroy the floating supported bilayers on silicon dioxide surface as well as those SLB formed on polymer-cushioned silicon surface. The surface of the solid support might exert some influence to the destruction of floating supported bilayers. However, a chemically grafted membrane layer, octadecyltrichlorosilane (OTS)-hybrid floating supported bilayer, is a perfect model bilayer to investigate the interactions between lipoplex and membranes. This membrane model had proven high stability to the exposure of solvents and lipoplexes over a considerable duration of time, suggesting the OTS-
floating supported bilayers were not destroyed by the lipoplexes. Their work has established a new floating supported bilayers model that has minimum interference from the solid support, and hence it is suitable for studying the lipoplex-membrane interactions and correlates the transfection efficiency of this gene-delivery vector [41].

The usefulness of floating supported bilayers as a physiological mimic to the bacterial outer membrane helps to encounter technical challenge to recreate and study the interactions of antimicrobial proteins in vitro. Clifton et al. [56] described the formation of asymmetric floating supported bilayers fabricated by sequential Langmuir-Blodgett deposition of 1-oleoyl-2-(16-thiopalmitoyl)-sn-glycerol-3-phosphocholine (thiolPC) and Langmuir-Schaefer deposition of floating supported bilayers consisted of lipopolysaccharides to mimic the bacterial outer membrane. The thiolPC layer and the floating supported bilayers are separated by a small water gap; hence the DPPC and lipopolysaccharides bilayers are floating on the solid support [56]. This floating-supported bilayer system has been fully studied and characterised using neutron reflectometry with high stability and reproducibility. The use of floating supported bilayers makes it possible to maintain the dynamicity, fluidity and translation freedom of the membranes; therefore, the membrane properties are not altered and behave similarly in their physiological environment [56–58]. Further studies show that lactoferrin, a cationic antimicrobial protein, directly interacts with lipopolysaccharides on the outer membrane via electronic interactions. The results from neutron reflectometry revealed a dramatic reduction in the floating supported bilayer coverage in the presence of lactoferrin, providing a structural picture of lactoferrin disrupting the outer membrane [56]. Another cationic enzyme, lysozyme was found to bind to the outer membrane electrostatically without changing the floating supported bilayer coverage, and a small increase in membrane bilayer roughness was also detected. These two studies both reported a change in floating supported bilayers properties upon interactions with the antimicrobial proteins; however, the neutron reflectometry data implied that the lysozyme disrupted the outer membrane in a different mechanism than lactoferrin [56].

5. Membrane proteins on gold-modified surface

Membrane proteins are well-known to be difficult to handle and study outside their natural lipid environment because they are flexible, unstable and relatively hydrophobic [1]. To overcome the problems, membrane proteins are extracted from membranes in the existence of detergents, which helps to stabilise membrane proteins during extraction, solubilisation, purification and crystallisation [1, 59]. To examine the structure-function relationships of membrane proteins, reconstitution of the artificial membrane environment is also essential in maintaining native protein configurations for biological functions [60]. Unfortunately, the detergents, which stabilise the membrane proteins, can easily disrupt the lipid bilayers (as described in Section 4) by penetrating the membranes and then form lipid micelles. Researchers are motivated to develop new approaches to encounter the technical difficulties in reconstituting the native environment for studying membrane proteins in vitro [61].
As with any structural or functional investigations with membrane proteins, the use of direct protein immobilisation on a solid support has been used intensively in the protein array and biosensors industry [62, 63]. Often the challenge for this method is that the immobilised proteins adapt into random orientations, causing a change in protein conformations and consequently reducing the bioactivity. Therefore, conformationally oriented immobilisation strategy should be considered to enhance the stability of the immobilised proteins. Site-directed mutagenesis, a protein-engineering method, is particularly useful to improve the quality of protein immobilisation on a solid support. The selected amino acid sequences of protein are precisely mutated and the modified amino acids are used for surface immobilisation [62, 63]. Two different approaches, cysteine and histidine residues mutations, have been developed as illustrated in Figure 4 [63–66]. The designed mutations (either cysteine or histidine) allow the membrane proteins to immobilise with a particular orientation in which their native conformations and activities are retained upon adsorption [14, 27, 67, 68]. Successfully, control protein orientation on a modified surface is a key to investigate the interactions and stereochemistry of multi-protein system [67].

![Figure 4. Protein immobilisation on gold (Au) or gold-modified surfaces: (a) direct immobilisation allows proteins oriented randomly, however, proteins orientation can be controlled by (b) thiol chemistry on cysteine residues (red) or (c) histidine (blue) interactions with nickel-nitrilotriacetic acid (Ni-NTA)-modified gold surfaces.](image)

5.1. Substituted cysteine in protein forms a gold-thiolate covalent bond

The cysteine-containing proteins can be directly immobilised on gold surfaces using thiol chemistry [69], i.e., the sulphur atom on the cysteine residue forms a strong gold-thiolate covalent bond with the gold surfaces [12]. The spontaneous deposition promotes the formation of a dense self-assembled monolayer on gold. The application of cysteine-thiol immobilisation is commonly associated with biosensors and arrays applications. Brun et al. [12] used bacterial outer membrane proteins as a model for protein assay, which has potential applications as scaffolds for tissue engineering, proteomics and diagnostics. The outer membrane protein A (OmpA) is a monomeric membrane protein that involves in the survival mechanism in *Escherichia coli* (*E. coli*) when exposed to environmental stresses. The beta-barrel transmembrane structure of OmpA spans though the bacterial outer membranes and plays a vital role in membrane stability [70]. In Brun and co-workers’ studies, OmpA was engineered by inserting a cysteine residue into the periplasmic turn, such that the OmpA was immobilised...
on gold surfaces in a specific orientation via the covalent gold-thiolate bond. The immobilised OmpA was circularly permutated in order to expose the c and n termini for the interactions with *Staphylococcus aureus* protein A (SpA), a pathogen bound to bacterium cell wall. The SpA bound to OmpA using a mutated B domain (named as Z domain) created an OmpAZ protein scaffold on the gold surface [70]. Neutron reflectometry was used to detect the addition of Immunoglobulin G (IgG) on the OmpAZ protein scaffold, and the thickness of each protein layers was determined in high resolution. This provided information on the orientation of IgG on the OmpAZ protein scaffold [70]. Furthermore, Brun and co-workers enhanced the detection of IgG by introducing a flexible linker as a functional motif to link two Z domains together on OmpA (ZZOmpA). The capacity of IgG binding to ZZOmpA was greatly enhanced and hence potentially increased the signal and sensitivity for protein array applications [71, 72]. The antibody protein array (IgG-ZZOmpA) has been further used to detect antigen influenza nucleoprotein (NP), that is highly conserved and the basis of diagnostic tests for influenza. The NP binding capacity as well as the structural orientation of the bound antibody-antigen layer, ZZOmpA-NP layer, were fully characterised [65]. Brun et al. [65] have demonstrated the potential of using neutron reflectometry as an *in situ* physical characterisation of the protein assay assembly process. The cysteine-orientated immobilisation technique enables the proteins to be used to maintain in their native structure in order to be fully functional as a protein scaffold for further applications such as antibody and antigen detections.

5.2. Histidine-tagged membrane protein binds to nickel-nitrilotriacetic acid

Another protein immobilisation strategy is based on the covalent interactions between histidine (His) residues and nickel-nitrilotriacetic acid (Ni-NTA) modified surfaces. The His residues are genetically engineered on either the N or the C terminus of recombinant proteins [63]. The nickel-nitrilotriacetic acid (Ni-NTA) functionalised gold surface has a high affinity to capture His-tagged proteins, and hence all bound proteins are conferred in a particular orientation [67]. The His-Ni-NTA affinity technique enables the proteins immobilise on a solid surface without altering their function, which has been a challenge in the development of protein assay for decades [63, 67]. The use of recombinant affinity tags has addressed the issues of protein orientation and surface density upon immobilisation on solid surface [67, 73].

Shen et al. [63] have reconstituted an artificial environment for the translocation and assembly module (Tam) nanomachine that catalyses the insertion and assembly of nascent membrane proteins into the outer membranes of bacteria. The TAM nanomachine is composed of two proteins: a beta-barrel contained TamA locates at the outer bacterial membrane and a beta-helical TamB spans in the inner bacterial membrane. The two protein subunits, TamA and TamB, are linked together by a three polypeptide-transport-associated (POTRA) domains in TamA that is located in the periplasm region. The precise movement and activity of the TAM nanomachines were studied using neutron reflectometry to provide an accurate measurement on the molecular movement of the Tam protein complexes [63]. Firstly, a hexa-histidine tag was specifically engineered into an extracellular loop of TamA to immobilise onto the Ni-NTA functionalised gold surface, followed by the addition of lipids for membrane reconstitution. The thickness of the membrane embedded beta-barrel, the three POTRA domains as well as
the hexahistidine extramembrane layers were separately determined in the reconstituted membrane environment. The architecture of the TAM complex (TamA and TamB) was also studied using neutron reflectometry; no structural movement was obtained upon interactions of TamB to the POTRA domains of TamA [63]. This study focuses on the interactions between a substrate protein, Ag43, and the TAM complex on a supported membrane. The presence of Ag43 triggered the POTRA domain of TamA to alter its conformational changes, projected away from the membrane. The movement of TamA created spatial constraint on the outer membrane and influenced TamB deposition within the Tam complex. Monitoring the TAM complex assembly pathways using neutron reflectometry allows further understanding on the operations of diverse cellular processes for virulence of bacterial pathogens [63].

6. Concluding remarks

Understanding the protein-membrane interactions is critical to expanding the applications in drug delivery, biological engineering and, especially, biosensors and microarrays [4]. In this chapter, examples of protein adsorption on various artificial membrane models are reviewed and discussed. Along with the technical advances of neutron reflectometry, information on molecular binding and environment and orientation of the lipids and biomolecules is investigated in great detail. The use of various artificial membrane models offers a methodological design suited to study protein/peptide-membrane interactions in different biological systems. The phospholipid monolayer models are particularly useful for biological applications exploring the influence of fluidity or surface properties [74]. The phospholipid monolayer consists of one leaflet of lipid, which represents only a half bilayer. The structural simplicity and versatility allow for monitoring accurately the lipid packing density [74]. Traditionally, the phospholipid monolayer is deposited at an air/water interface using the Langmuir-Blodgett method; the surface coverage of lipid monolayer at air/water interface can be easily controlled, enabling examination of the influence of phospholipid membrane properties on protein- and peptide-membrane interactions [18]. Alternatively, lipid can be deposited and functionalised using a polymer or surfactant at a solid/liquid interface [17]. The increasing availability of different artificial membrane models extends and improves the ability to mimic the biological equivalent.

Protein adsorption studies on various fluid phospholipid bilayers have been developed: (a) single supported lipid bilayers, (b) tethered lipid bilayer membranes, (c) polymer-cushioned bilayer membranes and (d) floating supported bilayers [29]. The ease of preparation and high stability of the support lipid bilayer make it the most popular artificial membrane model [29]. Tethered lipid bilayer membranes are complex interfacial structures where the phospholipid bilayers are deposited on molecular anchors that are chemically linked between the membrane and the solid support [47]. These tethered lipid bilayer membranes provide a thicker intermediate water layer that mimics the natural cell-like environment for membrane-associated process [74]. Fluid lipid membranes can be deposited on a soft, polymeric layer, named polymer cushioned membranes [16]. The incorporation of molecular anchors and polymers between membranes and solid surface improves the long-term stability of the phospholipids.
and provides a better biomimetic condition for the applications of membrane proteins [53]. Recently, floating supported bilayers are used as an advance membrane model to mimic the phase behaviour of lipids and the fluidity of bilayers in nature. The floating supported bilayers are created using a series of both Langmuir-Blodgett and Langmuir-Schaefer methods onto a solid support, and this is the most suitable system to replicate the asymmetry of bacterial outer membranes [55]. However, artificial membranes may not always be the most suitable system to investigate and examine the structural and functional aspects of membrane proteins because (a) lipid bilayers can be destroyed by the membrane protein solution that contains surfactant and (b) it is difficult to control the protein orientation on a solid support [60]. Thus, membrane proteins can be modified or mutated at desired locations to control the protein orientation when immobilised on gold-modified surfaces. Cysteine or histidine residues are the common approaches for the binding of protein onto thiol-modified or nickel-nitrilotriacetic acid-modified gold surfaces [63–66]. These conformationally oriented immobilisation strategies allow the membrane proteins to retain their native conformations and activities upon adsorption and also it creates a stable lipid layer surrounding the membrane protein. This review illustrates and highlights examples of the role of neutron reflectometry for studying proteins and peptides in biomimetic membranes. Many important advantages and unique features of neutron reflectometry offer numerous opportunities for innovative applications and technical development for in vitro study of biomolecules. Only a small number of neutron reflectometry applications in protein adsorption are demonstrated, the potential for studying protein-membrane interactions in multi-components should not be underestimated in the future [15].

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